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14. ABSTRACT Triptolide (TPL), a diterpenoid triepoxides-like molecule purified from <i>Tripterygium wilfordii</i> Hook F, is a potent anti-tumor agent. However, the effect of TPL on breast cancer is not well studied. In past year, we have successfully finished the following works: 1) demonstrated the effectiveness of TPL in inhibition of breast cancer growth in vitro; 2) examined the effect of TPL in anti-growth of breast cancer xenografts; 3) synthesized and preparation of RGD/NGRPA-TPL-lipo; 4) examined the inhibitory effect of RGD/NGR-PA-TPL-lipo on growth of tumor cells in vivo in CAM model. The effect of RGD/NGR-PA-TPL-lipo on breast cancer xenografts and its angiogenesis will be further studied.					
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INTRODUCTION

Most anti-tumor drugs are originally derived from naturally occurring biological active molecules from plants or micro-organisms. For example, Taxol comes from the Pacific yew (*T. brevifolia*). Along this research line of drug discovery, we are interested in Triptolide (TPL).

TPL is a diterpenoid triepoxides-like molecule ($C_{20}H_{24}O_6$) with a molecular weight of 360.4 Dalton (1-4). It is purified from *Tripterygium wilfordii* Hook F, a herb that has been used as a natural medicine in China for hundreds of years. Several studies have shown that TPL can block growth of tumor cells (5-10). While the action mechanism of TPL is largely unknown, a few studies have suggested that it may act via inducing apoptosis (11-12). We have found that TPL can significantly reduce the level of *c-myc* and two pairs of major cell cycle complexes: cyclin A/cdk2 and cyclin B/cdc2. On the other hand, TPL could activate caspase 3 and PARP (13). Our special finding is that TPL also reduces the activity of telomerase, an ideal target for cancer therapy (paper in preparation).

The clinical trials of TPL as anti-tumor agent have been carried out in China. As shown in Table 1, the results were remarkable. When the therapeutic effect of TPL was evaluated in 21 patients, 10 of these patients experienced complete remission (CR, 47.6%); while 5 underwent a partial remission (PR). The total effective rate was 71.4%. There were different rates of remission in different types of acute leukemia. The highest rate of complete remission was achieved with acute granulocytic leukemia (75%), which was better than any current chemotherapy regimens for this disease.

Table 1. Effect of TPL on Different Types of Acute Leukemia

Types of leukemia	Cases	CR	%	PR	%	NR	%	TR	%
Granulocytic	8	6	75.0	1	12.5	1	12.5	7	87.5
Mononuclear	7	3	42.9	2	28.6	2	28.6	5	71.5
Lymphocytic	3			1	33.3	2	66.6	1	33.3
Erythroleukemic	2	1	50.0			1	50.0	1	50.0
Megakaryocytic	1			1	100.0			1	100.0
Total	21	10	47.6	5	23.8	6	28.6	15	71.4

This initial clinical data strongly indicates that TPL has significant anticancer effects and thus may have potential for being developed into a novel anti-tumor agent.

However, the effect of TPL on the breast cancer has not been studied.

In the past year, we have carried out experiments using highly purified TPL. The results are summarized as following.

BODY

We have recently been able to obtain a large amount of highly purified, crystallized TPL (**Fig. 1**) and have started experiments to explore its potential as a therapeutic agent for treating breast cancers.

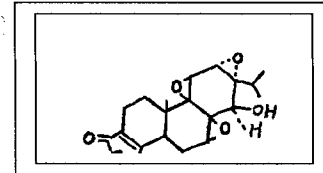


Fig. 1. Structure of TPL

I. Anti-Cancer Effect of TPL *in vitro*

In the initial experiments, the *in vitro* effects of TPL on tumor cell growth were examined. When 4T1 breast cancer cells were treated with 10--50 ng/ml of TPL overnight, the cells appeared to be sicker (more rounded and detached) than those treated with 100 ng/ml of Taxol, and their **proliferation** (evaluated by ^3H -thymidine incorporation) was reduced in a dose-dependent manner. Importantly, the effect of 2 ng/ml of TPL was approximately equivalent to 100 ng/ml of Taxol for both aggressive MDA-435 cells and 4T1 breast cells, indicating that TPL is a **very potent** anti-tumor agent. Furthermore, the ability of tumor cells to **form colonies** was also greatly inhibited by TPL. A statistical comparison showed that 100 ng/ml of Taxol, giving 60% inhibition of colony formation, was less effective than 2 ng/ml of TPL, which gave 80% inhibition rate. These results were very reproducible.

II. Effect of TPL on Primary Breast Cancer

Similarly, the *in vivo* effect of TPL was very promising. In one experiment BALB/c mice that had established 4T1 **primary** breast tumors (size 0.2 cm diameter) was i.p. injected with TPL at a low dose (4 μg /mice/day). After 14 days, the tumors were harvested, photographed and weighed. The results show that the TPL-treated tumors were reduced to 50% of the tumors that treated with vehicle-alone in control group ($P < 0.05$).

III. Improvement of TPL Formulation

In all of our *in vivo* studies, mice treated with TPL at 0.15 mg/kg/day (equivalent to 4 μg /mouse/day) for two weeks could well tolerated the TPL without obvious sickness signs or weight loss (data not shown). However, when BALB/c mice (syngenic mouse for 4T1 breast cancer) were treated with 0.27 mg/kg/day (equivalent to 7 μg /mouse/day) for two week, they showed some signs of sickness, indicating that an optimal dose should be carefully worked out before going to clinical trials.

Reutrakul and coworkers have reported that a modest anti-tumor effect was observed with an i.p. dose of 25 μg /mice every other day (3). The dose different between this study and our study may due to the purity of TPL. However, the higher dose of 50 μg /mouse three times weekly was lethal (3). It seems that the therapeutic window is relative small.

TPL possesses a small molecule weight (MW 360 Dalton), therefore, it is easy to randomly diffuse out of vessels and accumulated in major organs, such as liver and kidney, and cause the adverse side-effect (personal communication with physicians carrying the clinical trial in China).

In our hands, the therapeutic window ($\text{LD}_{50}/\text{ED}_{50}$) of TPL in mouse models is about 4. Although the currently available formulation may be probably adequate to initiate human clinical trials in the USA, we would prefer to develop TPL into a form that targets tumors with a relatively higher specificity than the free TPL.

One promising new approach is based of previous studies showing that liposomes can be used as a carrier for Doxorubicin. Liposomal Doxorubicin (DOXIL) at a dose of 25 mg/M^2 exerts an anti-cancer effect as potent as free Doxorubicin at 75 mg/M^2 , and more importantly, has a much lower toxicity (13-19). This formulation has already been approved by FDA and is widely used in cancer treatment. The rationale underlying this liposomal formulation is based on research showing that junctions between cells

in tumor blood vessels are looser and thus more leaky than normal vessels (22-25). While free Doxorubicin (MW of 543 Dalton) can easily diffuse into heart tissue, Doxorubicin that is encapsulated in liposomes with a diameter of about 100 nM can not easily diffuse out of normal blood vessels, but can diffuse into tumor sites via their much more leaky blood vessels (13-21).

The successful use of liposomal Doxorubicin (DOXIL) prompted us to make a related but somewhat different kind of liposome, RGD/NGR-PA-TPL-liposome, to see if it would be more specific for the target tumor cells and thus more potent and safer than free TPL.

RGD/NGR-PA TPL liposomes have two potential advantages over TPL alone. First, RGD/NGR-PA TPL is larger than TPL, which should increase the selectivity for leaking out of only those blood vessels in tumors. Secondly, it would specifically target on tumor endothelial cells because it is covered with two peptides, RGD and NGR, which bind preferentially to tumor cell specific surface proteins, $\alpha v \beta_3$ integrin and CD13.

RGD is a ligand of the $\alpha v \beta_3$ and $\alpha v \beta_5$ integrins that are expressed on surface of both tumor cells and tumor endothelial cells (26-29). While the RGD peptide alone has no detectable effect on tumor cells (30), peptides linked to RGD can target tumors via the RGD-integrin interaction on surface of tumor cells (27, 28). We anticipate that the internalization of RGD tagged TPL, through the $\alpha v \beta_3$ and $\alpha v \beta_5$ integrin-mediated pathway, may facilitate specific killing of tumor endothelial cells (27, 28), effectively reducing blood supply to the tumor.

NGR is a peptide ligand of aminopeptidase N (CD13) that allows one to target angiogenic blood vessels with NGR conjugated drugs. Immunohistochemic staining has shown that CD13 expression is up-regulated in human tumors, but is not detected in blood vessels of various normal tissues stained under the same conditions (32). The NGR peptide motif has already been used to deliver cytotoxic agents, such as pro-apoptotic peptides and tumor necrosis factor- α to tumor vasculature. (27, 28, 31). Thus, other drugs designed to interfere with tumor vasculature may also be more effective when coupled to an NGR peptide. (28).

Our idea is to combine the RGD and NGR peptide motifs into a single fusion polypeptide and then to conjugate TPL to this polypeptide in order to increase the specificity of delivering TPL to tumor vessels. The RGD motif was fused to the NGR motif via a glycine link domain. This fused-peptide should be able to bind to tumor or endothelial cells by either the RGD or the NGR motif, although we anticipate that the effects will depend on the number and the affinity of $\alpha v \beta_3$ and CD13 on cell surface.

This fusion polypeptide was further modified to take advantage of an approach recently reported in *Science* (33), which describes targeted nanoparticles (NP). In this approach The $\alpha v \beta_3$ ligand was covalently linked to a cationic lipid that formed so-called NP enclosing multiple mutant Raf genes. These liposomes targeted gene delivery to the tumor neovasculature and resulted in a sustained regression of established primary and metastatic tumor (33). We believe that if we conjugate TPL to RGD/NGR fusion peptides which also have a lipid tail that can anchor onto the outside of the bio-membrane of liposomes (Lipo), we will have generated a novel targeting liposome that not only is more selective for leaking out of tumor blood vessels, (because of its larger diameter), but also will bind to specific targets on the tumor neovasculature and tumor cells (via $\alpha v \beta_3$ integrin and CD13).

We propose to use PEG-DSPE (1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine-N-[Carboxy(Polyethylene Glycol)2000, Avanti Polar Lipids Inc.) to form the liposomes, because the PEG confers DSPE liposomes with a brush-like coating, which prevents the DSPE liposomes from nonspecific opsonization by plasma proteins. The PEG can also block recognition and clearance by macrophages and other elements present in the reticuloendothelial system. This approach should increase the half-life of liposomes in the circulation and should facilitate the accumulation of liposomes in tumors (34-37).

Our current four step protocol for preparing RGD/NGR-PA-TPL-Lipo is as following:

First, the RGD/NGR fusion peptide was synthesized with a lysine (R) and aspartic acid (D) C-terminal, which allows conjugation first to palmitic acid $\{CH_3-(CH_2)_{14}COOH\}$ and then TPL.

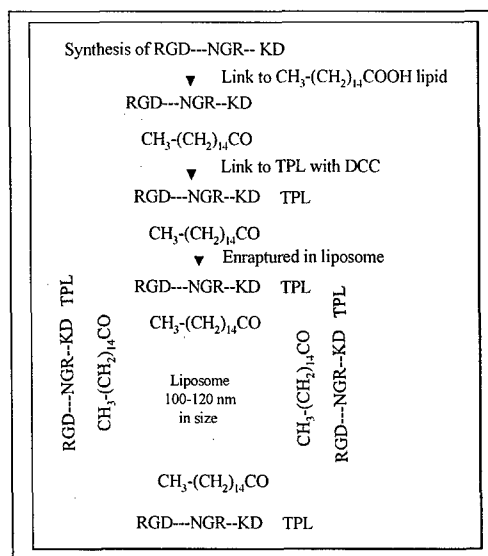
Second, the palmitic acid (PA) lipid was linked to the C-terminal Lysine (R) to generate RGD/NGR-PA.

Third, the RGD/NGR-PA was linked to TPL with N,N'-Dicyclohexylcarbodiimide (DCC) at the C-terminal aspartic acid residue (D) to form RGD/NGR-PA-TPL.

Fourth, the RGD/NGR-PA-TPL was mixed with PEG-DSPE liposome to form the targeting RGD/NGR-PA-TPL-lipo in a relatively uniform size (100-120 nm). When The PA lipid merged in the bio-lipid layer, the RGD/NGR head had a 50% chance of being on the outside surface of the liposome.

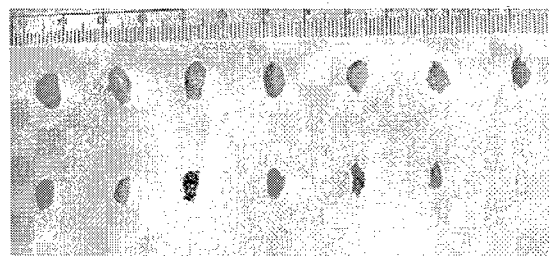
We finished small scale preparation of RGD/NGR-PA-TPL-lipo and tested its bioactivity. The preliminary result showed that RGD/NGR-PA-TPL-lipo could inhibit the growth of the proliferating endothelial cells, indicating that the conjugation process is feasible and the TPL activity keeps intact.

Since the RGD/NGR-PA-TPL-lipo was designed to preferentially target on tumor/endothelial cells, the appropriate testing system should be tumor bearing mice.



The effect of RGD/NGR-PA-TPL-lipo on tumor cells was tested first in chorioallantoic membranes (CAM) of 10 days-old chicken model, since it is a easy and fast *in vivo* system for the testing of anti-tumor agents. The tumor cells (5×10^6) were added to the CAM and allow cells to grow for two days to establish a visible tumor. Then, the tumors bearing egg were randomly divided into RGD/NGR-PA-TPL-lipo treated group or RGD/NGR-PA-TPL-lipo treated group. The agents were delivered by i.v. injection once. Five days later, the tumors were harvested, pictured and weighted. The result (left figure) showed that the tumors in RGD/NGR-PA-TPL-lipo treated group were smaller than that in the vehicle alone controls ($p < 0.05$).

Effect of Triptolide on the Growth of Tumor Cells in CAM model



Taken together, our results demonstrate that TPL, and possibly the TPL formulation described here could be useful as anti-tumor agents because: 1) TPL inhibits tumor cell proliferation, colony formation, tumor growth and tumor metastasis at extremely low concentrations; 2) its inhibitory effect is comparable to or better than four conventional chemotherapy drugs, Taxol (*in vitro*), Adrimycin, Cisplatin and Mitomycin (*in vivo*), and seems more potent than these drugs; and 3) it probably can be formulated in such way as to improve its therapeutic window by employing the novel modern drug deliver methods, such as the novel RGD/NGR-PA-TPL-lipo formulation described here.

Key Research Accomplishments

Taken together, **in past year**, we have successfully finished the following works: **1)** demonstrated the effectiveness of TPL in inhibition of breast cancer growth *in vitro*; **2)** examined the effect of TPL in anti-growth of breast cancer xenografts; **3)** synthesized and preparation of RGD/NGR-PA-TPL-lipo; **4)** examined the inhibitory effect of RGD/NGR-PA-TPL-lipo on growth of tumor cells *in vivo*.

Conclusions

TPL is a potent anti-breast cancer agent. The improved targeted form of TPL could enhance its therapeutic effect.

Reportable outcomes

(Due to or partially due to this support)

1. Shanmin Yang, Jinguo Chen, Charles B. Underhill, Sunghee Kim, Luping Wang, Xu-Ming Xu and Lurong Zhang: Effect of Triptolide on the Telomerase Activity in Human Cancer Cells. Proc. Annu. Meet. Am. Assoc. Cancer Res. 2004, 993
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